Chimeric Smooth Muscle–Specific Enhancer/Promoters
Valuable Tools for Adenovirus-Mediated Cardiovascular Gene Therapy

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Abstract—Gene transfer with adenoviral vectors is an attractive approach for the treatment of atherosclerosis and restenosis. However, because expression of a therapeutic gene in nontarget tissues may have deleterious effects, artery-specific expression is desirable. Although expression vectors containing transcriptional regulatory elements of genes expressed solely in smooth muscle cells (SMCs) have proved efficient to restrict expression of the transgene, their use in the clinical setting can be limited by their reduced strength. In the present study, we show that low levels of transgene expression are obtained with the smooth muscle (SM)-specific SM22α promoter compared with the viral cytomegalovirus (CMV) enhancer/promoter. We have generated chimeric transcriptional cassettes containing either a SM (SM-myosin heavy chain) or a skeletal muscle (creatine kinase) enhancer combined with the SM22α promoter. With both constructs we observed significantly stronger expression that remains SM-specific. In vivo, reporter gene expression was restricted to arterial SMCs with no detectable signal at remote sites. Moreover, when interferon-γ expression was driven by one of these two chimeras, SMC growth was inhibited as efficiently as with the CMV promoter. Finally, we demonstrate that neointima formation in the rat carotid balloon injury model was reduced to the same extent by adenoviral gene transfer of interferon-γ driven either by the SM-myosin heavy chain enhancer/SM22α promoter or the CMV promoter. These results indicate that such vectors can be useful for the treatment of hyperproliferative vascular disorders. (*Circ Res.* 2001;88:468-475.)

Key Words: smooth muscle myosin ■ creatine kinase ■ interferon-γ ■ restenosis ■ gene transfer

Smooth muscle cell (SMC) migration and proliferation are key factors in the development of atherosclerosis and restenosis after angioplasty or stent placement.[1] These processes are associated with phenotypic modulation of SMCs characterized by modification of gene expression.[2] Hence, the overexpression of an individual gene in vascular SMCs by local gene transfer is considered a promising therapeutic approach. Several vector systems have been developed to facilitate gene delivery into SMCs. Among them, the most widely used are recombinant adenovirus vectors (Ads). In SMCs, Ads have been used successfully to impair cell growth in vitro and to diminish intimal hyperplasia in animal models of restenosis.[3] However, because systemic administration of a high viral dose is associated with cytotoxic effects in nontarget tissues, the clinical application of Ads can be compromised.[4] To solve these problems, several strategies have been considered. A local delivery and the retargeting of the vectors may reduce viral dissemination. Moreover, modification of the expression cassette by addition of cell type–specific transcriptional elements may ensure targeted protein expression and reduce host immune responses to transgenic proteins.[5] The replacement of viral promoters by SM-specific *cis*-acting sequences has been studied previously.[6,7]

The transcriptional regulation of SM-specific genes has been centered on structural proteins (actin and myosin) and actin-binding proteins (SM22α and calponin).[8] The SM-myosin heavy chain (MHC), a major contractile protein and a powerful marker for the study of SMC differentiation, is regulated by multiple *cis*-acting elements, including CarG boxes, that have been shown to be key regulators for SM gene transcription.[9] SM22α, a calponin-related protein that is expressed specifically in SM, possesses 3 CarG boxes in its promoter that are sufficient to direct arterial tissue–specific expression.[6,10] The limiting factor for the use of tissue-specific promoters is the low level of expression compared with their viral counterparts. To our knowledge, no data related to the level of SM22α-driven gene expression are available in an adenoviral context. Hence, in the present study, we evaluated the strength of the SM22α promoter and revealed a low efficiency by comparison with the cytomegalovirus (CMV) promoter. Two strategies have been described to obtain stronger artificial tissue-specific promoters.

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Composition of Adenoviral Expression Cassettes

Adenovirus	Enhancer (Position)/Orientation	Promoter	Gene
AdCMVeGFP	...	CMV	eGFP*
AdSM22eGFP	...	SM22α[10]	eGFP
AdCK/SM22eGFP	CK (−919/−711)/antisense[13]	SM22α	eGFP
AdSM-MHC/ SM22eGFP	SM-MHC (−1332/−1225)/sense[12]	SM22α	eGFP
AdSM22LacZ	...	SM22α	LacZ
AdRSVLacZ	...	RSV	LacZ
AdCMVIFNγ	...	CMV	IFN-γ†
AdSM-MHC/SM22IFNγ	SM-MHC (−1332/−1225)/sense	SM22α	IFN-γ
Adnull	...	...	...

*Subcloned from pEGFP (Clontech).
†GenBank AF10466.

One consists of the construction of synthetic promoter libraries by random combination of tissue-specific regulatory elements.[11] Another is to exploit the endogenous genomic sequences that enhance tissue-specific expression. We have explored this latter strategy by fusing the SM22α promoter either with the SM-MHC enhancer or with the creatine kinase enhancer.[12,13] In vitro, we demonstrated that these new transcriptional cassettes were more effective than the SM22α promoter alone. In addition, they improved the cell type–specific expression of the reporter gene. In vivo, when injected intravenously in mice, no expression was observed in nontarget tissues. Finally, when tested in the rat carotid balloon injury model, the expression of interferon-γ (IFN-γ) leads to a biological effect when driven either by one of the enhancer/promoter combinations (SM-MHC/SM22α) or by the CMV promoter. Taken together, our data indicate that these chimeric transcriptional elements are excellent candidates for a targeted vascular gene therapy.

Materials and Methods

Cells and Culture Conditions

Rat SMCs were isolated from aortas (ratAo) and from injured aortas 15 days after balloon catheter deendothelialization (ratIT15).[14] The human pulmonary epithelial A549 and the murine myoblast C2C12 cell lines were purchased from the American Type Culture Collection (CCL-185 and CRL-1772; Manassas, Va). The rat intestinal epithelial cells (IEC18) were kindly provided by Dr Simon-Assmann (INSERM U381, Strasbourg, France). Cells were cultured in DMEM containing 10% FCS (Life Technologies) supplemented with 0.2 U/mL insulin (Sigma-Aldrich) for IEC18. During and after adenoviral infection, cells were maintained in 2% FCS–containing medium, except C2C12, which were maintained in 10% FCS to prevent fusion.

Adenovirus Construction, Production, and Titration

Expression cassettes were obtained by insertion in the adenoviral E1-deleted region of the different promoters and enhancers followed by an intron and a transgene, as listed in the Table. All viral vectors were constructed as infectious plasmids by homologous recombination in *Escherichia coli* BJ5183.[15] They were all deleted for the E1 and E3 regions. Adenoviral plasmids were digested by *Pac*I and transfected in the 293 complementation cell line. After virus propagation and purification, infectious units (iu) were titrated.

In Vitro Experiments

The susceptibility to adenoviral infection was determined for each cell type using various multiplicities of infection (MOIs) of AdCMVeGFP. Cells were infected at day 1 (D1) and harvested at D2, and the percentage of expressing cells was determined by quantitative analysis of enhanced green fluorescent protein (eGFP) expression by flow cytometry (FACSCalibur, Becton Dickinson Biosciences). In other experiments, cells were infected and harvested at D4 to allow eGFP accumulation. For the duration of expression experiment, cells were seeded at a density of 1.10^5 cells/well in 6 well plates (Falcon, Becton Dickinson) at D0 and infected with either AdCMVeGFP or AdSM-MHC/SM22eGFP at D1. They were maintained up to D15 in 2% FCS–containing medium without medium replacement and harvested at different time points to quantify eGFP expression. The strength of the different regulatory sequences was measured using the global fluorescence index (GFI) calculated as the product of the percentage of eGFP-positive cells by the mean fluorescence value.[16] For the cell-differentiation experiment, infections were performed as described above and then cells were washed and placed in either 2% FCS–containing or 10% FCS–containing medium and harvested at D4. For cell growth inhibition experiment, cells were seeded at a density of 3.10^4 cells/well in 6 well plates at D0 and infected with AdSM-MHC/SM22IFNγ and AdCMVIFNγ at D1. Cells were counted at D5. A sample of the culture medium was harvested at D4 to quantify the secreted rat IFN-γ (Quantikine M rat IFN-γ, R&D Systems).

In Vivo Gene Transfer Into Mice

All animal experiments were performed in a special pathogen-free facility and were conducted according to the French regulations for animal experimentation (Decret No. 87-848, 19.10.1987). Nine-week-old female immunocompetent mice were used for experiments (C57BL/6, Iffa-Credo, L'Arbresle, France). At D0, adenoviruses were injected intravenously at 2.10^9 iu. Mice were killed at D3, and organs (liver, lungs, spleen, and heart) were harvested and fixed in PBS containing 2% formaldehyde. The eGFP expression was evaluated by fluorescence microscopy.

In Vivo Gene Transfer Into Rat Carotid

Adult male Wistar rats (body weight 400g) were used for experiments (Iffa-Credo). The left common carotid artery was injured by balloon catheterization.[17] Then a 1-cm-long segment of the injured carotid was isolated and flushed with 0.2 mL NaCl 0.9%. Adenovirus (50 μL) (2.10^9 iu) was allowed to dwell for 5 minutes. Rats were killed at different time points according to the experiments. Vessels were fixed with either 2% or 4% formaldehyde in PBS at normal blood pressure. Then carotids were excised and treated for the different histological analyses.

Histological and Immunocytochemical Analyses

SM22α immunohistochemical staining was performed on 4-μm-thick sections. Deparaffinized sections were incubated with the E-11 SM22α mouse monoclonal antibody (kindly provided by Drs A. Chiavegato and S. Sartore, University of Padua, Padua, Italy) at a dilution of 1:100.[18] The presence of SM22α was revealed by means of the streptavidin-biotin-complex peroxidase method (LSAB kit, Dako). For β-galactosidase activity, vessels fixed in 2% formaldehyde were incubated for 24 hours at 37°C in 5 mmol/L $K_3Fe(CN_6)$, 5 mmol/L $K_4Fe(CN_6)$, 2 mmol/L $MgCl_2$, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Five-micrometer sections were examined for blue nuclear staining. For morphological analyses, 5-μm sections from 4% formaldehyde–fixed arteries were stained with H&E, and the medial and intimal areas were evaluated by image analysis using an Olympus DP11 camera and AnalySIS software (Soft Imaging System GmbH).

Statistical Analysis

All results are expressed as mean±SEM and were analyzed by Student's *t* test. Differences were considered statistically significant at values of $P<0.05$.

Results

In Vitro Evaluation of SM22α Promoter Strength and Specificity

The susceptibility to adenovirus infection of ratAo, ratIT15, IEC18, and A549 cells was determined by infection with AdCMVeGFP at MOIs ranging from 1 to 1000. The MOI corresponding to 100% of eGFP-expressing cells was determined by flow cytometry. A549 cells, IEC18, and ratIT15 but not ratAo were efficiently infected at relatively low MOIs (A549: MOI 50; IEC18: MOI 50; ratIT15: MOI 10; ratAo: MOI 300). These data revealed that SMCs have a different susceptibility to adenoviral infection according to their phenotype.

To better compare eGFP expression driven either by the SM22α promoter or the CMV promoter, the level of eGFP was analyzed by flow cytometry 3 days after infection. We observed that only a fraction of AdSM22eGFP-infected SMCs expressed the reporter gene. The fluorescence intensity of this fraction was determined, but we decided to express the results as the mean of fluorescence of the global population. This better reflects the total amount of transgene that can be produced by one expression cassette in a given cell type. Thus, the promoter strength was evaluated using the GFI.[16] We observed that the GFI of AdSM22eGFP-infected cells was stronger in ratAo than in ratIT15. A weak background was present in the 2 non–SM cell lines from rat intestine (IEC18) and human lung (A549) (Figure 1A). Interestingly, when compared with AdCMVeGFP (GFI arbitrarily set at 100%), the AdSM22eGFP-related expression was nearly identical in ratIT15 and ratAo (8±1.8% versus 5.7±0.7%, Figure 1B), reflecting a similar regulation of both promoters in a defined SMC population. However, the SM22α promoter–related expression remained relatively weak.

Influence of the MOI on SM22α Promoter–Driven Expression

The comparison of the CMV- and the SM22α-driven expression levels in SMC populations was done with different viral doses according to their susceptibility to adenoviral infection. We cannot rule out that the viral load influences the cell physiology and consequently the strength of the promoters. Such interactions were demonstrated in primary human SMCs.[19] To evaluate the effect of high viral loads on transgene expression, ratAo and ratIT15 were infected at an MOI higher than the viral dose that allowed 100% of infection. In ratAo, increasing the viral dose from MOI 300 to MOI 500 resulted in a 5-fold increase of the SM22α-driven expression, whereas the CMV-driven expression remained unchanged (Figure 2). In ratIT15, the CMV- and SM22α-driven eGFP expression increased with the MOI. Thus, the comparison of viral and SM-specific promoters should be done without superinfection, because they are not equally affected by the viral dose.

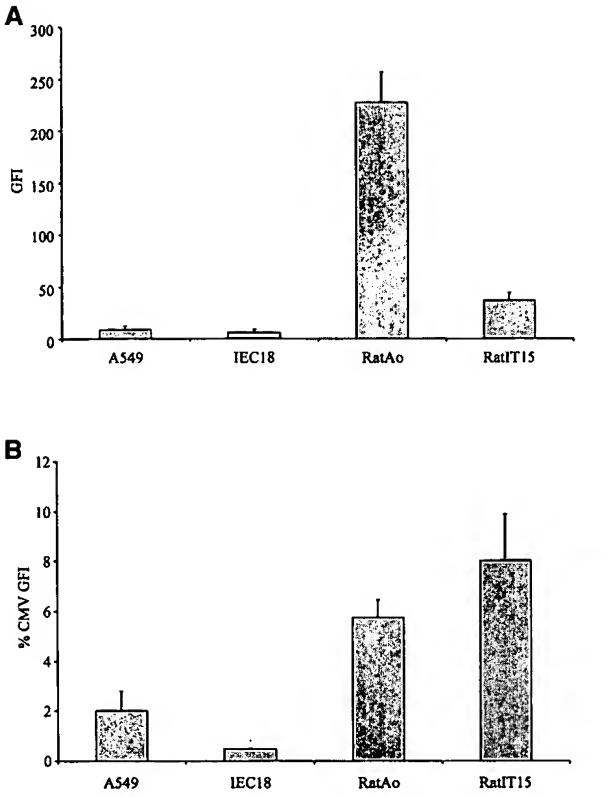

Figure 1. SM22α promoter strength in SMCs, IEC18, and A549 cells. Results are presented using the SM22α-related GFI (A) or as a percentage of the CMV GFI (B). Cells were exposed to either AdSM22eGFP or AdCMVeGFP, and the GFI was determined in each cell type at D4. Using the SM22α promoter, a significant expression was observed only in SMCs reaching 6% to 8% of the CMV strength. The levels in IEC18 and A549 were relatively low, as expected. Results are mean of 2 to 8 experiments.

Expression of the SM22α Protein During Neointima Formation in Rat Carotid Arteries

Like natural transcription units, the regulation of tissue-specific expression cassettes in the adenoviral context depends largely on the presence of the required transcription factors. We hypothesized that the regulation of the SM22α-driven expression cassette will depend on the regulation of the endogenous SM22α, because, like the SM-MHC or the SM α-actin, SM22α is a SMC transcriptionally regulated

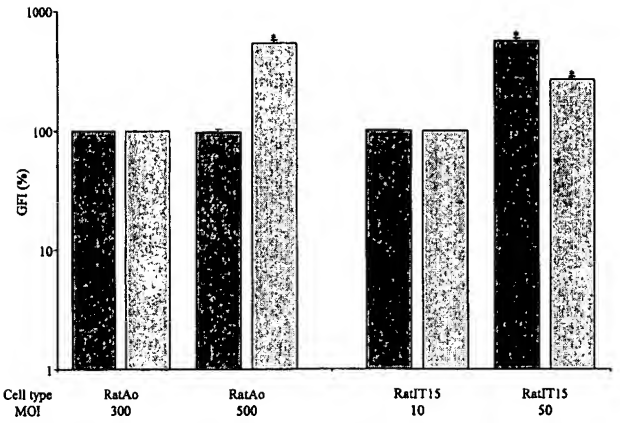

Figure 2. Impact of the MOI on the GFI in ratAo and ratIT15. Results are expressed as the percentage of a GFI obtained with 100% of infected cells. Cells were infected at D0 with either AdCMVeGFP (dark gray) or AdSM22eGFP (light gray) at indicated MOIs. In ratAo, the levels of expression increased with the MOI when AdSM22eGFP was used, whereas they remained stable with AdCMVeGFP. Inversely, the expression observed in ratIT15 increased with both AdSM22eGFP and AdCMVeGFP. Each experiment was performed twice (*$P<0.05$).

differentiation marker.[20] Therefore, we determined the pattern of expression of SM22α during intimal thickening formation in the rat model of carotid injury. In uninjured rat carotid, SM22α was present throughout the media (Figure 3A). After injury, we did not observe a downregulation of SM22α in the media at any time point analyzed (Figures 3B through 3E). SM22α was expressed during neointima formation in migrating and proliferating SMCs (Figures 3C through 3E). We therefore confirmed that the *LacZ* bacterial gene driven by the SM22α promoter could be expressed in this model of arterial injury. As previously reported,[6] we observed a blue nuclear staining in cells scattered throughout the media (Figure 3F).

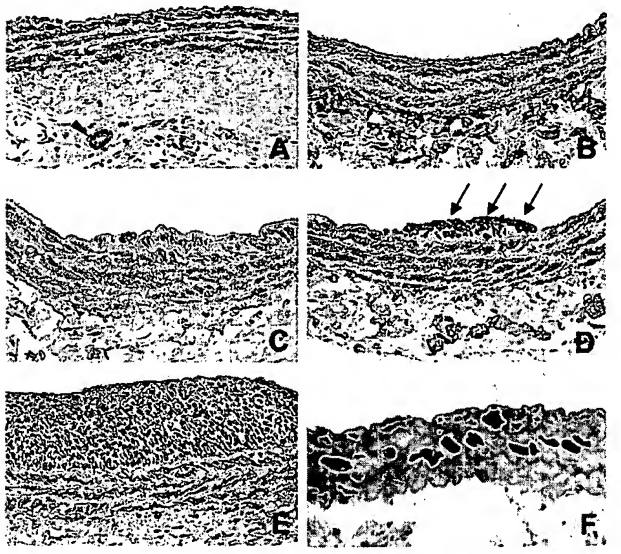

Figure 3. Endogenous SM22α protein expression and SM22α-driven *LacZ* expression after rat carotid endothelial injury. Uninjured (A) and injured carotids collected at D1 (B), D3 (C), D5 (D), and D9 (E) after balloon injury were stained with E-11 anti-SM22α antibody. Samples were counterstained with H&E. SM22α-positive cells (brown staining) were present in the media (A through E), the neointima (C through E), and in small vessels of the adventitia (A, arrowhead). A stronger staining was observed in the cell layer lining the lumen (D, arrows). Seven days after AdSM22 *LacZ* infection, the *LacZ* staining was observed in SMCs scattered in the media (F).

In Vitro Evaluation of the Strength and Specificity of Chimeric Regulatory Sequences

In vitro experiments revealed the weakness of the SM22α promoter and suggested that it could not drive production of sufficient amounts of therapeutic molecules in a clinical setting. This assertion is reinforced by the rather low efficiency of infection reported with classical catheter-based gene delivery techniques. Improvements of promoter strength may be achieved by the addition of enhancers from viral, cellular, or synthetic origin.[11,21] We decided to use enhancers belonging to the second group.

The creatine kinase enhancer (CKenh) was chosen for its known muscle specificity and the SM-MHC enhancer (SM-MHCenh) for its SM specificity. The strength and specificity of expression of these chimeric regulatory elements were tested in ratAo, ratIT15, and A549 cells. The chimeric constructs were more specific than the SM22α promoter alone, because eGFP expression was 4-fold weaker in A549 cells with both the CKenh/SM22α and SM-MHCenh/SM22α promoters (Figure 4A). In addition, all SM22α-based promoters gave similar results in IEC18 (data not shown). The CKenh increased the level of expression by about 3-fold in ratAo and ratIT15, with 13.2±5% and 21.3±3% of the CMV-related expression, respectively. The SM-MHCenh showed the same pattern of expression except that it was slightly stronger (not statistically significant) in both SMC populations, with 16.9±4% and 23.5±2.8% of the CMV-related expression.

Because of the skeletal muscle origin of the CKenh, we tested the corresponding chimeric vector in murine myoblasts using the C2C12 cell line. Surprisingly, we did not observe a strong expression with the CKenh/SM22α promoter. The background attributable to the SM22α promoter (1.7% of the CMV promoter) was increased by the CKenh (5.6%), but the expression level remained 3 to 4 times lower than in SMC populations (data not shown).

Thus, in vitro experiments indicate that the CKenh/SM22α and the SM-MHCenh/SM22α expression cassettes increase transgene expression and maintain specificity.

Effect of Cell Differentiation on Chimeric Promoter-Related Expression

Because it was reported that serum concentration has an impact on the SM22α promoter–driven *LacZ* expression in plasmid transient transfections,[10] we evaluated the effect, with Ad, of serum-induced SMC dedifferentiation on SM22α promoter–driven, CKenh/SM22α promoter–driven, and SM-MHCenh/SM22α promoter–driven expression. AdCMVeGFP was used as positive control. Only ratAo cultured in 2% or 10% FCS were infected, because it is well accepted that ratIT15 are undifferentiated in reduced-serum conditions.[22] We observed that the SM22α promoter–driven,

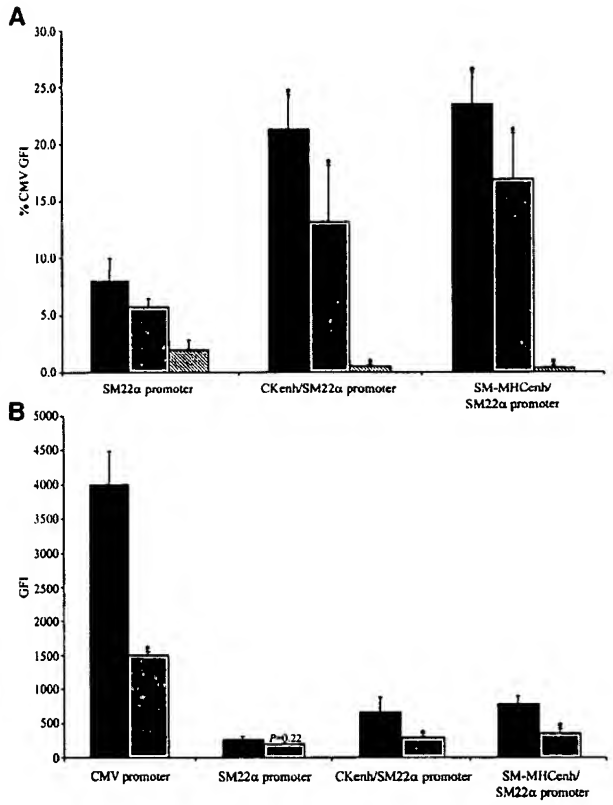

Figure 4. A, Evaluation of strength and specificity of chimeric promoters. Results are presented as a percentage of the CMV GFI. RatIT15 (black bars), ratAo (gray bars), and A549 cells (hatched bars) were exposed to SM22α promoter–containing vectors and AdCMVeGFP at the appropriate MOI. The addition of the CKenh or the SM-MHCenh increased the SM22α promoter–related expression 3-fold in ratAo and ratIT15, whereas this expression decreased in A549 cells. Results are mean of 4 to 8 experiments (*$P<0.05$). B, Impact of the differentiation on SM22α promoter–related expression. RatAo were exposed to either AdCMVeGFP or SM22α promoter–containing vectors at MOI 300 and cultured in DMEM containing 2% FCS (black bars) or 10% FCS (gray bars) for 3 days. The differentiation state of ratAo had a similar effect on the different promoters. A stronger expression was observed in more differentiated cells. Each experiment was performed twice (*$P<0.05$).

CKenh/SM22α promoter–driven, and SM-MHCenh/SM22α promoter–driven eGFP expressions were decreased in high serum concentration (Figure 4B). This result might suggest that the chimeric promoter containing two SM-specific regulatory sequences (SM-MHC and SM22α) follows, such as the SM22α promoter alone, the normal regulation of the corresponding differentiation markers, known to be more highly expressed in differentiated cells. However, the muscle-specific CKenh did not modify the high-serum-induced downregulation of eGFP expression when associated with the SM-specific SM22α promoter. More surprisingly, the CMV-driven expression, when tested in both culture conditions, was also regulated by serum concentration. Taken together, these data indicate that the strength of viral, muscle, and SM-specific regulatory sequences is similarly downregulated after SMC dedifferentiation.

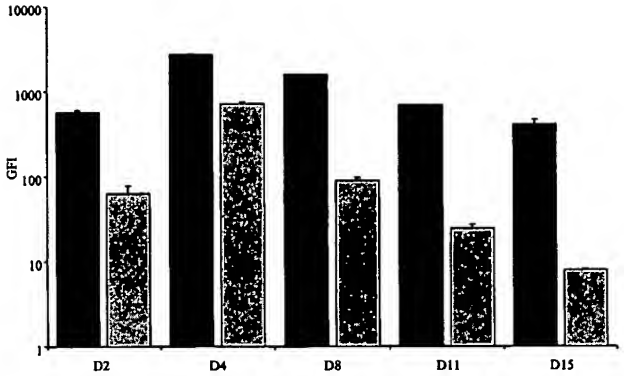

Figure 5. Duration of the expression using the SM-MHCenh/SM22α promoter. RatAo were exposed to AdCMVeGFP (black bars) or AdSM-MHC/SM22eGFP (gray bars) at MOI 300 at D0 and cultured in DMEM containing 2% FCS during 15 days. Cells were harvested at different time points to analyze the GFI. The expression reached a maximum at D4 with both promoters followed by a rapid decrease when driven by the SM-MHCenh/SM22α promoter compared with the CMV promoter. Variations between each time point were statistically significant for a defined promoter. The experiment was performed twice.

In Vivo Evaluation of Chimeric Vectors in Mice

Dissemination of adenoviral vectors after intravenous or intra-arterial administration is a major problem for their use as therapeutic gene vehicles. Therefore, we verified that SM22α promoter–driven expression did not occur in any of the main adenovirus natural target organs (ie, liver, lungs, spleen, and heart). This experiment was done by adenovirus injection into C57BL/6 mice for technical facilities. AdCMVeGFP and Tris-HCl injections were used as positive and negative controls. Four mice were injected intravenously with 2.10^9 iu of each construct, and the presence of eGFP expression in the different organs was determined by fluorescence microscopy. The Southern blot performed on the DNA extracted from the 4 main organs indicated that all mice were correctly infected (data not shown). The 4 mice injected with AdCMVeGFP showed a significant fluorescence in all organs tested, whereas mice injected with SM22α-containing vectors or the two chimeric promoter-containing vectors did not show any fluorescence, confirming, therefore, the tissue specificity of these constructs (data not shown).

Duration of the Expression Using AdSM-MHC/SM22eGFP

Even if similar results were obtained with both chimeric constructs in terms of strength, specificity, and regulation, we decided to concentrate on the SM-specific cassette. Before studying the efficacy of a combination between this sequence and a therapeutic gene, we addressed the question of the duration of expression driven by the SM-MHCenh/SM22α promoter in vitro in ratAo. Indeed, the high proliferation rate of ratIT15 does not allow their use in long-term experiments, because they rapidly become confluent even in low-serum conditions.[22] After a maximum reached at D4, the expression decreased with both the SM-MHCenh/SM22α and the CMV promoters, albeit at different rates (Figure 5). However, 35% of the cell population still expressed the eGFP at D15 when driven by the SM-MHCenh/SM22α promoter, confirming,

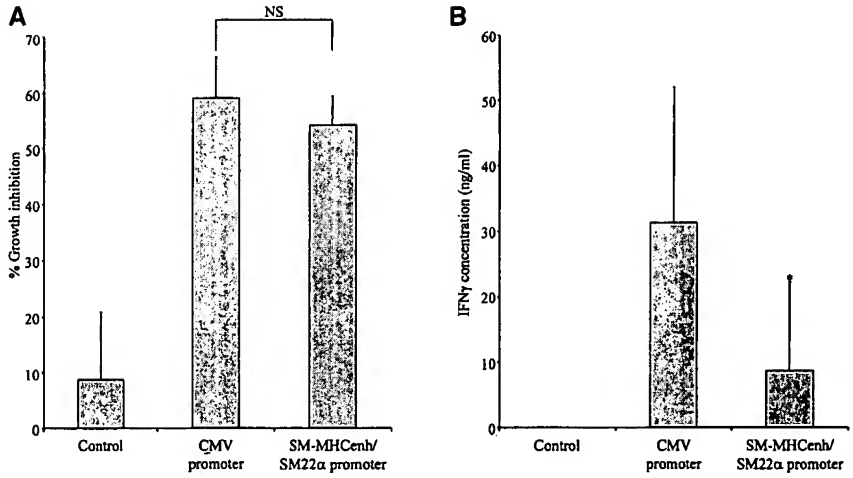

Figure 6. Effect of rat IFN-γ driven by different promoters on ratIT15 proliferation. Cells were exposed to AdCMVIFNγ, AdSM-MHC/SM22IFNγ, or AdCMVeGFP (control) and counted at D5. The antiproliferative effect of IFN-γ (A) was correlated to rat IFN-γ concentration analyzed by ELISA (B). There were no significant differences between both IFN-γ vectors (P=0.37), whereas IFN-γ concentration was 3- to 4-fold higher (P=0.026) using the CMV promoter. Results are mean of 4 experiments.

therefore, the interest of this sequence to drive a SM-specific expression cassette.

Effect of AdSM-MHC/SM22IFNγ on SMC Proliferation In Vitro

We analyzed the capacity of the rat IFN-γ gene driven by the SM-MHCenh/SM22α promoter to prevent SMC proliferation. We used the ratIT15, which better represents the target cells in the treatment of restenosis. AdSM-MHC/SM22IFNγ was compared with AdCMVIFNγ and with AdCMVeGFP used as negative control. Results are expressed as the percentage of growth inhibition 4 days after infection.

The chimeric promoter generated a 54±5.2% growth inhibition versus 59±7.3% for the CMV (Figure 6A). The difference is not statistically significant (P=0.37). The SMC growth inhibition attributable to AdCMVeGFP was small (9±12.1%) and not statistically significant (P=0.41) compared with noninfected cells.

Supernatants of infected cells were harvested at D4 and analyzed by ELISA to determine IFN-γ concentration. The SM-MHCenh/SM22α promoter gave rise to an IFN-γ concentration of 8.7±13.5 ng/mL versus 31.2±20.8 ng/mL (P=0.026) for the CMV promoter (Figure 6B). No detectable levels of IFN-γ were obtained in noninfected and AdCMVeGFP-infected cells.

Effect of AdSM-MHC/SM22IFNγ on Neointima Formation in the Rat Carotid Model

We finally examined the effect of AdSM-MHC/SM22IFNγ in the rat model of carotid injury. Rats were locally injected with 2.10^9 iu of AdSM-MHC/SM22IFNγ, AdCMVIFNγ, Adnull, AdCMVeGFP, and AdSM-MHC/SM22eGFP subsequently to balloon catheter injury, and arteries were analyzed after 14 days. In Adnull-, AdCMVeGFP-, and AdSM-MHC/SM22eGFP-treated control rats, extensive intimal thickening was observed in all injured vessels with no statistically significant differences (0.106±0.023 mm², 0.090±0.021 mm², and 0.085±0.018 mm², respectively Figures 7A and 7D). In contrast, AdCMVIFNγ- and AdSM-MHC/SM22IFNγ-treated rats showed a similar 25% to 35% reduction in the cross-sectional area of the carotid neointima (0.070±0.051 mm² [Figure 7B] and 0.057±0.009 mm² [Figure 7C], respectively). The media was not affected by either adenoviral vector treatments (0.113±0.011 mm² and 0.109±0.013 mm² versus 0.119±0.015 mm², 0.114±0.013 mm², and 0.115±0.014 mm², respectively).

Discussion

It has been previously demonstrated that adenoviral vectors could be transcriptionally targeted to vascular SMCs.[6] However, the use of tissue-specific promoters is a matter of debate, because they are generally considered to be weak. Thus, there is still a need to develop efficient, strong, and specific SM regulatory sequences allowing the production of therapeutic proteins in sufficient amounts. In the present study, we show that a chimeric construct containing a SM-specific enhancer associated with the SM22α promoter meets these criteria. We first confirmed that the SM22α promoter alone is relatively weak, reaching only 8% of the CMV-promoter strength. Previous studies indicate that the SM22α promoter in a plasmid context was as strong as the RSV promoter.[10] This is consistent with our unpublished observations, showing that the Rous sarcoma virus (RSV) promoter is about 10% as strong as the CMV promoter. However, it is important to note that results obtained with adenoviral vectors may vary according to SMC origin. We observed that ratAo are less sensitive to adenoviral infection but more capable of expressing transgenes under the control of the SM22α promoter than ratIT15. When these SMC populations are exposed to a SM22α-containing adenovirus, only a fraction of infected cells expresses the reporter gene, suggesting that SMC populations are heterogeneous as far as their capacity of transactivating this SM-specific promoter is concerned. We also observed a heterogeneous response to infection using the viral CMV promoter, because increasing the viral load generated stronger expression only in ratIT15. It has been shown elsewhere that nuclear factor-κB, known to be an important transcription factor required for CMV promoter activity, is present in SMCs involved in response to balloon injury.[23]

Because the SM22α is a transcriptionally regulated differentiation marker, we hypothesized that the expression cassette and the endogenous promoter would be simultaneously activated in vivo.[20] In contrast to the SM22α downregulation

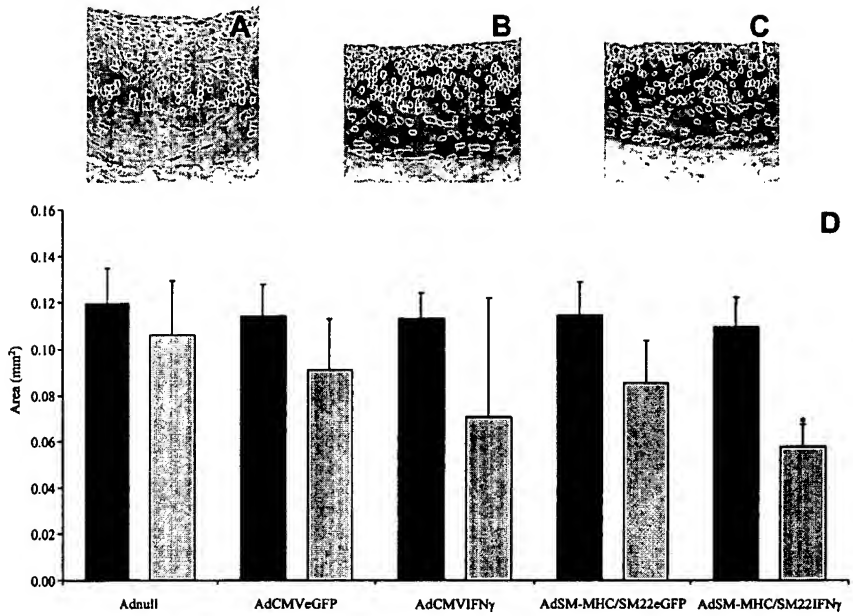

Figure 7. Inhibition of neointimal thickening using Adnull (A), AdCMVIFNγ (B), AdSM-MHC/SM22IFNγ (C), AdCMVeGFP, and AdSM-MHC/SM22eGFP. After balloon catheter injury, rat carotids were infused for 5 minutes with 2.10^9 iu of each adenoviral solution (n=5 to 6). Rats were sacrificed at D14. Medial (black bars) and neointimal (gray bars) areas were measured by image analysis (D). The IFN-γ–containing vectors led to a similar decrease in intimal thickening. This decrease was significant with the chimeric promoter but not with the CMV promoter compared with Adnull (*P=0.002, P=0.18), AdCMVeGFP (*P=0.015 and P=0.43), and AdSM-MHC/SM22eGFP (*P=0.022 and P=0.59), respectively. Medial area remained unchanged.

reported in the rabbit model, the endogenous protein was present at all time points in both medial and neointimal SMCs of the injured rat carotid.[18] These data confirm the interest of an SM22α promoter–driven expression cassette for sustained in vivo expression in synthetic and contractile SMCs.

Despite interesting properties, such as in vitro and in vivo cell lineage–restricted expression, the SM22α promoter remains weak. Therefore, we improved the expression cassette using the SM-MHC and the CK enhancers. They were both efficient despite their heterologous origin, rabbit for SM-MHCenh and human for CKenh, and increased the SM22α promoter–related expression about 3-fold. These enhancing effects were in the same range as those observed with other promoters.[12,24] In addition, we observed that these enhancers maintained the specificity of expression and did not change the SM22α promoter regulation, because the stronger transcriptional activity was obtained with the more differentiated cells.

To our knowledge, this is the first time that the CKenh has been tested in SMCs alone or in combination with a SM-specific promoter. It is surprising that the CKenh, usually defined as a striated muscle-specific enhancer, had a SM-specific effect. We could hypothesize that adenoviral sequences have an impact on the CKenh and that they redirect its specificity. Such interactions between viral sequences and a specific enhancer/promoter chimera containing the CKenh were previously described.[24] On the other hand, the CKenh contains functional domains involved in the regulation of genes from skeletal, cardiac, and SM origin. These muscle-specific motifs could be responsible for the activation of the CKenh in an SM context. Indeed, the SM22α promoter CarG boxes lie closer to the CKenh CarG motif, because we placed the enhancer in an antisense orientation shown to be responsible for a stronger expression.[13] It has already been shown that SM-selective expression depends on the positioning of CarG elements and cooperative interactions between them.[25] Confirming this hypothesis, no expression was observed in skeletal muscle after intramuscular injection in mice of the CKenh-containing construct. Taken together, these results indicate that both chimeric constructs are potentially useful for vascular gene transfer strategies. However, we chose the SM-MHCenh/SM22α promoter for its strict SM-specific sequence composition.

The duration of gene expression, studied with this regulatory sequence, revealed a rapid decrease of the eGFP expression compared with the CMV promoter–driven expression. This decrease could be explained at least in part by the low serum condition used to prevent SMC proliferation. It was recently demonstrated that the SM22α promoter and SM-MHC promoter presented a 8-fold loss of their activity in long-term serum-deprived SMCs compared with serum-fed cells. On the contrary, the activity of a viral promoter was unaffected by serum culture conditions.[26] Interestingly we observed that 35% of the AdSM-MHC/SM22eGFP-infected cells still expressed the eGFP 14 days after infection. In addition, the absence of downregulation of the endogenous SM22α during the intimal thickening process additionally supports the idea that conditions encountered in vivo will favor the persistence of the expression driven by the SM-MHCenh/SM22α promoter.

The therapeutic interest of the chimeric promoter was then tested in combination with the rat IFN-γ known for its antiproliferative properties.[27] We observed a significant inhibition of neointimal SMC growth in vitro and a reduced intimal thickening formation in the rat carotid model. This adenoviral-mediated local expression avoids the administration of huge amounts of recombinant protein, which is usually associated with side effects. For instance, a 50% reduction of the neointima requires a 100 μg/kg IFN-γ treatment during 7 days.[27]

The CMV-driven IFN-γ effect was surprisingly not significant compared with Adnull, AdCMVeGFP or AdSM-MHC/SM22eGFP (P=0.18, P=0.43, and P=0.59, respectively). Several factors, such as toxicity and inflammation, could be

responsible for the substantial variations observed in this group. We may hypothesize that the CMV promoter produces toxic IFN-γ concentrations, leading to cell death and to an inflammatory response that could increase the number of infiltrating cells in the neointima and thus partly counteract the beneficial effect of IFN-γ. By comparison, the lower IFN-γ concentration produced by the SM-MHCenh/SM22α promoter could favor the persistence of expressing cells, resulting in a more reproducible inhibition of the neointima.

In conclusion, the present study demonstrates that chimeric-specific regulatory elements based on the SM22α promoter are efficient for the treatment of cardiovascular hyperproliferative disorders. The development of new generations of adenoviral vectors containing these chimeras could be one of the promising strategies for a SMC targeted gene therapy devoid of side effects.

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